Levels of Alpha- and Beta-synuclein Regulate Cellular Susceptibility to Toxicity from Alpha-synuclein Oligomers.

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Short Title: Alpha-synuclein oligomer toxicity is enhanced by copper.

Key words: synuclein, oligomer, toxicity, FoxO3a, iron, Parkinson's disease

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Non-Standard Abbreviations

α-syn – alpha-synuclein

β-syn – beta-synuclein

γ-syn – gamma synuclein

α-syn-TO – toxic oligomers of α-syn

Δ2-9 - mutant of α-syn or β-syn lacking the amino acid residues 2-9

Δ2-9/H50A – mutant of α-syn lacking the amino acid residues 2-9 and with the histidine residue a position 50 mutated to alanine.

Δ2-9/H65A - mutant of β -syn lacking the amino acid residues 2-9 and with the histidine residue a position 65 mutated to alanine.

1-100 – mutant of α-syn or β-syn lacking the residues after 100.

AD - Alzheimer's disease

DBD – DNA binding domain

FoxO3a – Forkhead box O3a

SNCA – gene locus of α-syn

HA – hemagglutinin

H50A - histidine residue a position 50 mutated to alanine in α-syn

H65A - histidine residue a position 65 mutated to alanine in β-syn

PD - Parkinson's Disease

p-FoxO3a – phosphorylated FoxO3a

T-FoxO3a – all forms of FoxO3a

ThT - thioflavine T

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ABSTRACT

Alpha-synuclein is associated with a range of diseases including Parkinson's disease. In disease alpha-synuclein is known to aggregate and has the potential to be neurotoxic. The association between copper and alpha-synuclein results in the formation of stellate toxic oligomers that are highly toxic to cultured neurons. We have further investigated the mechanism of toxicity of alpha-synuclein oligomers. Cells that overexpress alpha-synuclein showed increased susceptibility to the toxicity of the oligomers while those that overexpressed beta-synuclein showed increased resistance to the toxic oligomers. Elevated alpha-synuclein expression caused an increase in expression of the transcription factor FoxO3a. Inhibition of FoxO3a activity by the overexpression of DNA binding domain of FoxO3a resulted in significant protection from alpha-synuclein oligomer toxicity. Increased FoxO3a expression in cells was shown to be caused by increased ferrireductase activity and Fe(II) levels. These results suggest that alpha-synuclein increases FoxO3a expression due to its intrinsic ferrireductase activity. The results also suggest that FoxO3a plays a pivotal role in the toxicity of both Fe(II) and toxic alpha-synuclein species to neuronal cells.

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INTRODUCTION

Alpha-synuclein (α-syn) is associated with a number of neurodegenerative diseases, including Parkinson's (PD), dementia with Lewy bodies, the Lewy body variant of Alzheimer's disease (AD) and multiple system atrophy. Fibrillar aggregates of α-syn are the main constituent of Lewy bodies and Lewy neurites associated with these diseases (1, 2). Extracellular α-syn is present as aggregates in both the *substantia nigra* of PD patients (2) and senile plaques of AD brains in the form of the non-Aβ component, NAC (3, 4). Clear links between α-syn and neurodegeneration have been found. Neuronal cell loss and Lewy body-like inclusions occur in animal models overexpressing α-syn (5) and the rescue of dopaminergic cells from death occurs following down-regulation of α-syn expression in the *substantia nigra* of a Parkinson's disease rat model (6). Inherited mutations in familial cases of PD also illustrate the importance of α-syn to pathology. Inherited cases are linked to both point mutations (7, 8) leading to single point changes in the protein sequence (eg. A30P, E46K, A53T) and triplication of the α-syn gene (SNCA) locus (9).

The prevalence of fibrillar aggregates of α-syn associated with neurodegenerative diseases has led many authors to hypothesise that the aggregates cause cell death (1, 10, 11). However, the survival of neurons with intracellular Lewy bodies shows that the presence of intracytoplasmic α-syn aggregates is not toxic to all cells (2). Considerable evidence suggests that oligomers, formed as prefibrillar intermediates, may be the toxic component (12-

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14). In addition, there is evidence that extracellular α-syn is neurotoxic. Recombinant α-syn, which readily assembles into filaments *in vitro* with similar morphology, staining and structure to α-syn filaments extracted from diseased brains, is toxic to cells when added to the culture medium, particularly in its aggregated form (15-22). The higher levels of α-syn oligomers in CSF of PD patients support a hypothesis that extracellular α-syn oligomers may be neurotoxic (23). However, the toxic species and mechanism of toxicity are still unclear.

While there is considerable and extensive evidence for the role of aggregates of α-syn in a variety of diseases, there is less evidence for its normal cellular role. There are currently two theories about the function of α-syn. The first suggests that it mediates the release of dopamine (24), while the second and more recent suggests it enzymatically reduces iron (25). There is considerable evidence that α-syn binds to copper and iron (25-30). α-syn has been shown to be a ferrireductase both *in vivo* and *in vitro* and it has also been shown to have reduced activity in Parkinson's disease (31). Detail kinetics studies have shown α-syn ferrireductase activity is regulated by substrate inhibition, is membrane associated and the active form is a tetramer (32). Overall, there is strong evidence for a link between α-syn and iron metabolism.

There is compelling evidence for a role in many neurodegenerative diseases for the loss of homeostasis of the redox active transition metals iron and copper and

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the resulting oxidative stress. High levels of copper, zinc and iron are found in and around amyloid plaques in AD brains (33). In PD brains, high levels of iron and zinc are found in the *substantia nigra* (34) and high levels of copper in the cerebrospinal fluid (35). While α-syn binds to copper and iron (25-30), α-syn aggregation is also stimulated in the presence of these metals (26, 29, 36). This has led us to examine whether the toxicity of extracellular synuclein proteins is exacerbated in the presence of metals. We have shown that the toxicity of α-syn aggregates increases in the presence of metals, in particular copper (37). This effect was not replicated with α-syn homologs, β-synuclein (β-syn) or γ-synuclein (γ-syn). The toxicity is caused by unique stellate soluble α-syn oligomers formed through morphological change in the presence of copper. Our findings suggested that oligomerisation of α-syn combined with a loss of metal homeostasis may be a key to the neurodegeneration observed in these diseases.

While initiation of cell death is a critical point in understanding the possible role of α-syn in cell loss in diseases like Parkinson's disease, cell death execution is possibly more interesting for potential intervention. From this point of view, the Forkhead box (FoxO) transcription factor family has gained increasing prominence both in the study of aging and neurodegeneration (38, 39). The relevance of FoxO3a to the study of α-syn and Parkinson's disease has recently been shown by two papers. The first demonstrated increased and ectopic expression of FoxO3a in PD brains (40). The second study using transgenic rats demonstrated that the level of α-syn induced neuron loss in the substantia nigra

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was increased by increase FoxO3a expression or reduced by overexpression of the DNA binding domain of FoxO3a (41). Therefore understanding the potential role of FoxO3a in α-syn toxicity is of great importance.

In the current study we found that FoxO3a plays a major role in toxicity induced by aggregated α-syn. Levels of expression of FoxO3a were modulated by levels of iron, which were dependent on ferrireductase activity of cellular α-syn. We found that the relative expression levels of both α-syn and β-syn play a critical role in susceptibility of SH-SY5Y cells to the toxicity of exogenous α-syn oligomers. These findings may have important implications for neuronal loss in PD and other neurodegenerative diseases.

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MATERIALS AND METHODS

Reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Purification of synuclein proteins

Expression and purification of recombinant α-syn and its mutations was as previously described (30). Using pET expression vectors in BL21 E. coli cells, untagged synuclein protein expression was induced at OD_{600} 0.5 – 1.0 with 1 mM IPTG for 4 hours. Cells were harvested by centrifugation (8000 x *g*) and lysed mechanically in 20 mM Tris-HCl/1 mM EDTA/pH 8.0 (Buffer A), 1 mM PMSF and 50 µg/mL DNase. Streptomycin sulfate was added to a final concentration of 1% to the lysate solution then centrifuged at 8000 x g. Ammonium sulfate (0.295 g/mL) was added to the supernatant (15% w/v solution) and stirred at 4 ºC for at least 1 hour. After centrifugation at 10,000 x g, the pellet was resuspended 50 mL Buffer A. The semi-purified lysate solution was loaded onto a 50 mL Q Sepharose column (Amersham Biosciences). The column was washed with 100ml Buffer A, then 2 column volume isocratic elution step to 25% Buffer A + 1M NaCl (Buffer B). Synuclein proteins were eluted with a broad gradient elution (10 column volumes) from 25% Buffer B to 50% Buffer B (all synuclein proteins eluting at approximately 350 mM NaCl). SDS-PAGE analysis of Q Sepharose fractions was performed and fractions enriched for synuclein were pooled. Synuclein proteins were collected as flow through from a PM30 cellulose membrane (Millipore) then concentrated with a PM10 PES membrane (Millipore).

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Purified synuclein proteins were dialysed extensively at 4ºC in Chelex-treated MilliQ. Protein concentration was measured by absorbance at 275 nm with extinction coefficient 5600 $M^{-1}cm^{-1}$ for α-syn (and mutants) and β-syn.

Production of synuclein fibrils

500 µl of 20 µM synuclein proteins in 10 mM Tris pH7.4 were aliquoted into 1.5ml screw-capped tubes. $CuCl_2$ was added to a final concentration of 100 µM. After incubation at room temperature for 45 minutes, tubes were laid flat on an orbital shaker and incubated at 37ºC shaking at 600 rpm for six days. Formation of fibrils was monitored using thioflavine T (ThT). 5 µl of each sample was mixed with 95 µl 10 µM ThT in 10 mM Tris pH7.4. Increased ThT fluorescence, indicating increased β-sheet structures, was monitored in 96 well plates with the FLUOstar Omega (BMG Labtech).

Cell culture

SH-SY5Y (human neuroblastoma) cells were cultured in 45% DMEM/45% Ham's F12 (LONZA) supplemented with 10% FBS, and pen/strep. Cells were maintained at $1x10^6/75cm^2$ at 37ºC and 5% CO_2 in a humidified incubator. The neuronal status of SH-SY5Y cells was monitored by RT-PCR with primers for tyrosine hydroxylase (TH), dopamine transporter (DAT) and vesicle monamine transporter 2 (VMAT2).

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Cell lines derived from SH-SY5Y cells and overexpressing either α-syn, β-syn or mutations of either were developed by stable transfection of plasmids (pCDNA3.1) containing the ORF of either protein using Fugene (Promega). The cell lines generated were as previously described (42).

MTT assay

For toxicity experiments, cells were plated at $2x10^5$ cells/well of a 24 well plate in DMEM (LONZA) supplemented with 10% FBS, pen/strep and grown overnight. Cells were treated for 48 hours with recombinant proteins at different concentrations. The MTT (3, [4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) reagent was resuspended at 2.5mg/ml in water. Medium and treatments were removed from the wells and 0.5 ml 50µl of MTT in HANKS solution was added per well and incubated for 30 minutes. After removal of the MTT solution, cells and the resulting reduced tetrazolium were solubilised in 800µl DMSO per well. Readings were taken at 570 nm using FLUOstar Omega (BMG Labtech). Each treatment was conducted in triplicate, averaged, then represented as percentage of untreated control (vehicle alone). Each experiment was repeated 3-5 times.

Western Blotting

Cells were lysed in 0.5% Igepal CA-630 and 'Complete' protease inhibitor cocktail (Roche), sonicated 3 x 3 seconds on ice, and centrifuged 10 000 xg for 3 minutes to remove insoluble membranes. Protein concentration was determined with a Bradford protein assay (Bio-Rad), according to the manufacturer's

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instructions. Protein concentrations were normalized and boiled for 5 minutes with 1 x Laemmli SDS-PAGE buffer. Samples were loaded into either a 10% (for FoxO3a) or a 12% (for α-syn) acrylamide SDS-PAGE gel, with a buffer of Tris (250 mM) + Glycine (1.92 M) + SDS (0.1% w/v), run at 250V and 35A for 45 minutes. Separated proteins were transferred to a PVDF membrane by a semi-dry transfer apparatus, run at 25 V and 100A for 1.5 hours. Membranes were blocked in 5% w/v non-fat milk powder in TBS-T for 30 minutes, incubated with primary antibody for 1-2 hours, and washed 3 x 5 minutes in TBS-T. Membranes were blocked again and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. A further 3 x 10 minute washes were performed, and the membranes developed with Luminata Crescendo or Luminata Forte ECL substrate (Thermo Scientific), and imaged with a Fusion SL CCD imaging system (Vilber Lourmat).

Rabbit monoclonal anti-α-synuclein (MJFR1, Abcam, immunogen human α-synuclein 1-150) was used for human α-synuclein detection at a dilution of 1:4000. Total FoxO3a (75D8) and phosphor-FoxO3a (ser253) were detected with rabbit antibodies at 1:1000 (Cell Signaling Technology). Mouse monoclonal anti-α-tubulin (T5186, Sigma, immunogen acetylated tubulin from *Strongylocentrotus purpuratus* sperm axonemes) was used at a dilution of 1:10,000. Mouse monoclonal, anti-dopamine transporter (AB2231, Millipore) was used at 1:1000 dilution.

Iron and ROS Assays

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The assay for iron concentrations in cell lines was as previously described (25). Basically, a commercial kit (Abcam) was used, following the manufacturer's instructions, to measure Fe(II) and total iron from a confluent T75 cell culture flask of each cell type per assay point. Reactive oxygen species (ROS) were measured utilising the fluorescent indicator, CM-H2DCFDA, as previously described (43). Cells were plated in 96-well plates at 75% confluency and returned to the incubator overnight. Medum was removed from test wells, and replaced by 50 μL of 5 μM probe in PBS, and incubated in the dark at 37ºC for 20 min. Probe was removed from the cells and replaced by 100 μL of pre-warmed DMEM. Fresh ferrous sulphate at a final concentration of 20 μM was added to four wells per experiment, and fluorescence intensity was measured using a microplate reader Fluostar Omega (BMG) with excitation and emission wavelengths of 488 and 534 nm, respectively, at time 0, 1 h, and 2 h. The change in fluorescence of treated cells compared to untreated cells was used as a measure of ROS generated by Fe(II) in the treated cells over time.

Statistics

Statistical analyses were conducted using a two-tailed Student's t test, statistical significance at *p*-value of < 0.05. Data are expressed as the mean ± standard error (S.E.M.).

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RESULTS

Synucleins and Exogenous Oligomer Toxicity

There has been considerable interest in the toxicity of α-syn oligomers when applied from outside the cell (44). We have previously shown that toxic oligomers of α-syn can be generated by reacting recombinant α-syn protein with copper (37). These stellate oligomers were found to be highly toxic to neuronal cells when compared to fibrils or oligomers prepared in the absence of copper. We therefore wished to further understand the mechanism of action of these stellate oligomers. We looked at the impact of increased cellular expression synuclein expression on the toxicity of recombinant α-syn oligomers applied exogenously. For clarity, the toxic α-syn oligomers will be referred to as α-syn-TO while the protein overexpressed in SH-SY5Y cells will be referred to by the protein's full name.

Stable cell lines overexpressing one of the three main synucleins were prepared by transfection of SH-SY5Y cells with pCDNA3.1 containing the open reading frame of either human α-synuclein, β-syn or γ-syn. The increased expression was confirmed by western blot as previously described (42). The stable cell lines were then treated with wild-type α-syn-TO at various concentrations. Survival of the treated cells was then determined. As can be seen in Figure 1A, α-syn-TO was toxic to SH-SY5Y cells transfected with the empty vector (pCDNA3.1) in a concentration dependent manner. In comparison, cells overexpressing γ-syn showed no significant difference in the response to α-syn-TO when compared to

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the controls. However, cells overexpressing α-synuclein were significantly more sensitive to α-syn-TO at concentrations between 0.5 and 5.0 μM while cells overexpressing β-syn were more resistant to the toxicity 5.0 μM and above. These results suggest that the cellular levels of both α- and β-synuclein influence the toxicity of α-syn-TO.

Increased expression of α-synuclein in cells resulted in increased sensitivity to α-syn-TO. We wished to determine if this effect could be altered by mutations in α-synuclein. We therefore produced stable cell lines expressing a range of structural mutations of α-synuclein. α-syn-TO was applied to these cell lines at a range of concentrations in parallel with cells expressing wild-type α-synuclein or the empty vector (pCDNA) as described above (Figure 1B). The mutations included a point mutation of the one histidine to an alanine (H50A). This mutation had no significant effect on the toxicity of α-syn-TO when compared to the effect on cells expressing WT α-synuclein. We also included deletion mutations of both the N- and C-terminus. Deletion of the C-terminus (1-100) also had no significant effect on the toxicity of α-syn-TO. In contrast, deletion of the N-terminus (Δ2-9) did have significant effect on the toxicity of α-syn-TO when compared to the effect on cells expressing WT α-synuclein. It was reduced for concentrations 0.5 and 5.0 μM. However, the toxicity at these concentrations was still significantly higher than the toxicity to control cells (pCDNA). Lastly, we also tested the toxicity of α-syn-TO on cell expressing a double mutation of both Δ2-9 and H50A (Δ2-9/H50A). In this case the toxicity of α-syn-TO to these cells was not

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significantly different to that of the toxicity to control cells. This implies that the mutation Δ2-9/H50A abolished the effect of overexpressed α-synuclein that increased cell sensitivity α-syn-TO toxicity.

We also wished to assess whether similar structural changes would alter the toxicity of α-syn-TO. We therefore generated recombinant protein for α-synuclein and generated aggregated protein through the same method as wild-type α-synuclein. The mutant forms of α-syn-TO generated were applied to SH-SY5Y cells for 48 h and the viability measured and compared to the toxicity of wild-type α-syn-TO (Figure 2A). A deletion from the C-terminus (Δ119-126) had no significant effect on the toxicity of α-syn-TO. However, the point mutation H50A significantly reduced toxicity at concentrations between 2.0 and 7.5 μM but not at higher concentrations. In contrast, the N-terminal mutation Δ2-9 abolished toxicity. We assessed the ability of these mutations to form aggregates using a ThT assay (Figure 2B). The ThT assay verified that all proteins used in these studies were able to form aggregates. Therefore the differences in toxicity were not due to differences in ability to aggregate.

α-synuclein and FoxO3a

FoxO3a is a transcription factor associated with cell death in neurodegenerative diseases. There is good evidence that increased expression of active FoxO3a increases neuronal loss in transgenic rats overexpressing α-synuclein (41). We wished to determine if the increased cell loss seen in our model was related to a

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change in FoxO3a expression. We grew SH-SY5Y cells either overexpressing wild-type α-synuclein or its mutant Δ2-9/H50A and control cells transfected with the empty vector (pCDNA3.1). Protein extracts were prepared from the cells and western blotting and immunodetection were performed. Blots were analysed for the expression of total FoxO3a, phospho-FoxO3a and tubulin as a loading control (Figure 3). The results showed that total FoxO3a but not phospho-FoxO3a were elevated in cells overexpressing wild-type α-synuclein but not the mutant Δ2-9/H50A. The lack of altered expression of the phosphorylated form is indicative that the change in expression is not due to an increase in the inactive form of FoxO3a. The lack of increased expression of FoxO3a in the mutant Δ2-9/H50A as opposed to wild-type α-synuclein correlated with their relative susceptibility to α-syn-TO toxicity.

FoxO3a is a transcription factor, as such it acts via binding to DNA. Inhibition of FoxO3a activity can be achieved through prevention of DNA binding. We therefore created SH-SY5Ycell lines that overexpressed either wild-type FoxO3a (FoxO3a-WT) or the DNA binding domain of FoxO3a (FoxO3a-DBD). Either the ORF of FoxO3a or the DNA binding domain (encoding 139 amino acids without the transactivation domain, residues 138-277) were cloned into pCDNA3.1. Both constructs also include an HA tag (hemagglutinin) for easy detection of the overexpressed protein. Cells were transfected with the constructs and stable cell lines selected by exposure to G418. Once overexpression in the cells was verified (data not shown) the cell lines and controls were treated with wild-type α-

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syn-TO and survival assessed after 48 h (Figure 4). Cells overexpressing FoxO3a-WT showed a slight but significant increase in susceptibility to α-syn-TO toxicity when compared to toxicity to pCDNA control cells. In contrast cells overexpressing FoxO3a-DBD were strongly and significantly protected from α-syn-TO toxicity. These results suggest that FoxO3a plays an important role in cell death caused by α-syn-TO. These results suggest that elevated FoxO3a expression induced by α-synuclein is the cause of the increased susceptibility of SH-SY5Y cells to α-syn-TO toxicity.

FoxO3a and Iron Reduction by α-synuclein

Having established a role for FoxO3a in cell death induced by α-syn-TO and that increased expression of FoxO3a is related to increased expression of α-synuclein it is important to try and identify how α-synuclein increases FoxO3a expression. We have shown previously that α-synuclein possesses ferrireductase activity and causes an increase in cellular reduced iron (Fe(II)) (25). Therefore we set out to determine if altering iron levels in cells would alter FoxO3a expression. SH-SY5Y cells were treated with 50 µM ferrous sulphate in serum free conditions for 24 hours. Extracts were then prepared from the cells and untreated controls and western blotting for FoxO3a levels carried out (Figure 5a). Treatment with Fe(II) resulted in a significant increase in FoxO3a expression when compared to controls. Additionally, we treated α-synuclein overexpressing cells with the iron chelator, deferiprone (250 µM) for 24 h under serum free conditions. Deferiprone is a strong, cell permeable Fe(II) chelator. We then

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measured the level of expression of FoxO3a using western blot (Figure 5B). Deferiprone significantly decreased FoxO3a expression when compared to controls cells treated only with the vehicle (DMSO). These results suggest that the levels of Fe(II) might alter the expression of FoxO3a.

We wished to test whether another ferrireductase could alter FoxO3a expression. A known mammalian ferrireductase is Steap3 (45) was cloned into pCDNA3.1 and overexpressed in SH-SY5Y cells. We then determined whether total and phospho-FoxO3a levels were altered in these cells. In parallel, we also looked at FoxO3a expression in cells overexpressing β-syn, which has no ferrireductase activity (25). Cells overexpressing Steap3 showed significantly increased levels of total FoxO3a but reduced levels of phospho-FoxO3a while those overexpressing β-syn did not (Figure 6). These results suggest that overexpressing a ferrireductase can alter FoxO3a expression but overexpression of another synuclein does not. We then tested the toxicity of α-syn-TO to Steap3 overexpressing cells (Figure 7A). When compared to the survival of control cells, α-syn-TO was more toxic to Steap3 overexpressing cells. Therefore, another known ferrireductase overexpressed in cells leads to increased FoxO3a expression and increased sensitivity to α-syn-TO. This is similar to the effect of α-synuclein overexpression. When combining these findings with the data mentioned above on iron and FoxO3a expression, then the most likely explanation for the alteration in FoxO3a levels is due to the increased levels of Fe(II) generated through iron reduction by α-synuclein.

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β-synuclein and **α-syn-TO toxicity**

Having elucidated a possible mechanism for how α-synuclein enhanced the toxicity of α-syn-TO we wished to look further at how β-syn can have the opposite effect. We used a battery of β-syn structural mutants which include deletion of the N-terminus (Δ2-9) or the C-terminus (1-100), point mutation of the single histidine (H65A) and a double mutation of the N-terminus (Δ2-9/H65A). Stable cell lines were generated overexpressing each of these mutants. The cell lines were then treated with α-syn-TO and compared to cell lines overexpressing wild-type β-syn of controls cells transfected with the empty vector (pCDNA). Survival of the cell lines was measured after 48 h with an MTT assay (Figure 7B). Neither the C-terminal mutant or the point mutation has any significant effect on the toxicity when compared to wild-type β-syn. However, the N-terminal mutation did alter the toxicity significantly, reducing the protective effect of β-syn. In comparison the mutant Δ2-9/H65A had an even stronger effect. There was no significant difference in toxicity between Δ2-9/H65A and pCDNA at any point, suggesting that this mutation abolished toxicity.

We have shown that β-syn has no effect on FoxO3a levels. Therefore, it is unlikely that the protective effect of β-syn against α-syn-TO toxicity is related to altered FoxO3a levels. We have previously shown that β-syn reduced the level of Fe(II) present in cells overexpressing the protein (25). We therefore looked at the levels of Fe(II) in both β-syn overexpressing cells and the mutant Δ2-9/H65A

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which has no protective effected against α-syn-TO toxicity. Figure 8A shows that overexpression of β-syn decreases Fe(II) levels in cells but overexpression of Δ2-9/H65A has no significant effect. The presence of Fe(II) in cells is known to potentially increase generation of ROS. Therefore we also measured ROS levels in cells with and without exposure to exogenous Fe(II). Overexpression of β-syn caused a decrease in the levels of ROS detected in SH-SY5Y cells, while overexpression of the mutant Δ2-9/H65A did not (Figure 8B). Therefore, the protective effect of β-syn against α-syn-TO toxicity may be due to a reduction in cellular sensitivity to ROS generated by Fe(II). As α-synuclein is a known ferrireductase (32) that increases Fe(II) levels in cells, this fits well with β-syn's known role as antagonistic to pathological changes caused by α-synuclein.

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DISCUSSION

The research presented here provides evidence for significant advancements in the understanding of the toxicity of oligomeric species of α-synuclein. First, toxicity is mediated through increased activity of FoxO3a. This protein is very strongly associated with cell death pathways and has been previously shown to be increased in the Lewy Bodies of PD patients (40). Second, increased expression of α-synuclein causes increased sensitivity to oligomer toxicity. This is likely due to increased expression of FoxO3a in the α-synuclein overexpressing cells. Third, increased β-syn expression has a protective effect against oligomer toxicity. This fits well with numerous reports that β-syn has a protective role against the negative effects of increased α-synuclein expression. This may be as a result of interaction with α-synuclein (46-49) or other means (50).

The involvement of FoxO3a in cell death in α-synuclein overexpression has been demonstrated in a transgenic rat model (41). In this model pathological effects of α-synuclein transgenic overexpression are reversed by inhibition of FoxO3a activity. This inhibition was induced by co-expression of the dominant negative inhibitor of FoxO3a activity, namely the DNA binding domain of FoxO3a. Our results support this role as we used a similar construct, overexpressed it in SH-SY5Y cells and showed that this inhibited the toxicity of α-synuclein toxic oligomers. In contrast overexpressing wild-type FoxO3a had no such effect. In this case it is possible that the extra FoxO3a did not increase the amount of active (non-phosphorylated) FoxO3a that entered the nucleus. Alternatively, a

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second factor may also be necessary for α-syn-TO toxicity which would remain limiting despite the increased levels of FoxO3a. Either way this data suggests that FoxO3a activity is necessary for α-syn-TO toxicity.

FoxO3a expression in SH-SY5Y cells was shown to be increased by the over-expression of α-synuclein or Steap-3 but not β-syn. A mutant of α-synuclein (Δ2-9/H50A) also had no effect on FoxO3a expression. This implies that the mutation alters α-synuclein sufficiently that its overexpression no longer impacts the expression of FoxO3a. In each case increased FoxO3a expression was not accompanied by increased phospho-FoxO3a expression, confirming that the increase is not just elevated accumulation of inactive FoxO3a (51). The increased expression of FoxO3a correlated with higher susceptibility of SH-SY5Y cells to α-syn-TO toxicity. In other words overexpression of α-synuclein and Steap-3 both increased FoxO3a expression and susceptibility to α-syn-TO toxicity but for Δ2-9/H50A and β-syn there was no increase in FoxO3a and no observed increase in α-syn-TO toxicity. These results also support the suggestion that FoxO3a expression mediates the toxicity of α-syn-TO. These findings in combination with the previous work in transgenic rats implie that FoxO3a is of major significance in terms of neuronal death mediated by α-synuclein.

It is also important to consider the mechanism by which FoxO3a activity/expression is increased. Both α-synuclein and Steap-3 show

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ferrireductase activity while Δ2-9/H50A and β-syn do not (25, 45). The implication is that the common factor resulting in increased FoxO3a expression is the generation of elevated levels of Fe(II), the product of reactions catalysed by ferrireductases. We verified this finding by demonstrating that increased iron caused an increase in FoxO3a expression in SH-SY5Y cells. The relative change in p-FoxO3a was much less implying that the increase was active FoxO3a. We showed the relevance of this finding for our system by applying an iron chelator to SH-SY5Y cells overexpressing α-synuclein and observing a reduced expression of FoxO3a. The change resulted in a high level of p-FoxO3a when compared to the total, indicating the reduction was in active FoxO3a. Therefore the fundamental factor which both regulates FoxO3a and susceptibility of SH-SY5Y cells to the toxicity of α-syn-TO may be Fe(II).

While our findings are novel, there have been a number of studies relating increased iron levels with increased activity of FoxO family transcription factors (52, 53). One of these papers implies that the increase of FoxO3a is mediated by the PI3K/AKT pathway. It has also been shown that iron overload increases FoxO3a expression (54). It is well known that FoxO3a expression is increased under oxidative stress and Fe(II) is readily able to catalyse reactions that increase oxidative stress such as the Fenton reaction (55). Iron-induced oxidative damage has frequently been shown to be mediated through the PI3K/AKT pathway (56-58). Therefore it is possible that the increased active FoxO3a seen

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in our findings is a result of downregulation of the PI3K/AKT pathway caused be oxidative stress from Fe(II).

These findings suggest that elevated cellular Fe(II) caused by overexpression of α-synuclein increases cellular susceptibility to the toxicity of oligomers of exogenous α-synuclein. Previous studies have also provided evidence that iron and α-synuclein can act in concert to cause cell death (59, 60). This is of considerable interest because of the possible role of this mechanism in diseases such as PD. Neuronal loss in the *substantia nigra* is the hallmark of the disease. PD patients show both elevated levels of α-synuclein and increased levels of Fe(II) (61). There is significant history of the relation of altered iron levels and PD but there has never been a causal connection established between elevated iron and loss if dopaminergic neurons in PD (62-67). However, one study in transgenic rodents suggests that iron chelation reduced pathological changes caused by α-synuclein overexpression (68) and a further study suggests that iron chelation also protected against the toxicity of 6-hydroxy dopamine in a mouse model (69).

Ambivalence exists as to whether α-synuclein is genuinely toxic *in vivo* and the form of the toxic species is also in question. There is strong evidence that α-synuclein can be toxic especially when overexpressed or introduced exogenously (70, 71). For some time toxicity was thought to come from fibrils but this has largely been dismissed in favour of the “toxic oligomer” hypothesis (72). There

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are large number of contenders for the mechanism of action of these (44). We developed a method to generate highly toxic oligomers by reacting recombinant α-synuclein with copper during a shaking process (37). The oligomers generated were stellate and their toxic action was not dependent on the copper required for their formation. However, there are other kinds of oligomers such as the pore forming variety (73, 74). While no one could claim to have generated “the” toxic oligomer that best models an *in vivo* oligomer, we feel our oligomer model is a good one because of its unique nature, reproducibility of toxic profile, high toxicity and ability to induce changes seen *in vivo* (such as those described in this report).

We showed in our data that β-syn overexpression protects against the toxicity of α-synuclein oligomers. As this is the opposite to the effect of overexpression of α-synuclein it further emphasises the potential role of β-synuclein as a regulator of the activity of α-synuclein. Expression of both α-synuclein and β-syn are regulated by similar pathways and transcription factors (75). This system exists probably to ensure levels of the two proteins are in balance. This balance would then protect against problems such as aggregation and toxicity of α-synuclein. Expression patterns and levels of α-synuclein and β-syn most closely overlap (3). β-syn is the most abundantly expressed synuclein in the brain, comprising 75-80% of the total mRNA of the synucleins (76). In both the mouse brain and the human *substantia nigra*, α-synuclein mRNA decreases and β-syn mRNA increases with age (77). In contrast to control patients, there is a dramatic

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increase in α-synuclein and decrease in β-syn mRNA levels in the *substantia nigra* of PD, DLBD and a Lewy body variant of AD patients (76). The importance of the balance between levels of the synucleins is highlighted by the observation that β-syn inhibits α-synuclein aggregation *in vitro* and *in vivo* (78-80). In addition, β-synuclein prevents aggregated α-synuclein from inhibiting the 26S proteasome (81). These functions lead to expression of β-syn in transgenic α-synuclein mouse models ameliorating neurodegenerative alterations, decreasing Lewy Body formation and preventing motor deficits (82), (78).

The nature by which β-syn exerts these effects is not understood. However, it is possible that it occurs through direct interactions. Both synucleins are able to form dimers and tetramers (83, 84) and the latter is believed to be the “correct” form for cellular α-synuclein. Therefore it is possible that heterotetramers exist which include both synucleins and which lack activities such as the proposed ferrireductase activity (25). We have previously demonstrated that cells overexpressing β-syn have lower Fe(II) levels than control cells (25). It is possible this reduced level of Fe(II) protects cells when exposed to α-syn-TO. However, this is not reflected in a change in FoxO3a as levels were not reduced in cells overexpressing β-synuclein. We showed that a mutant form of β-synuclein (Δ2-9/H65A) did not have the same effect as wild-type β-synuclein in protecting cells from α-syn-TO toxicity. While cells overexpressing β-synuclein showed both lower levels of intracellular Fe(II) and Fe(II) generated ROS, Δ2-9/H65A did not. This may be because it is unable to interact with α-synuclein or

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unable to be incorporated in synuclein tetramers. We have shown that membrane associated tetramers of α-synuclein are the form that has iron reducing activity (32). If the mutant Δ2-9/H65A has reduced ability to inhibit this activity, cells would generate more Fe(II). As we have shown Fe(II) can increase FoxO3a levels which mediates the toxicity of α-syn-TO.

In conclusion we have demonstrated that FoxO3a plays a pivotal role in the toxicity of α-synuclein oligomers. Inhibition of its activity blocks toxicity. Overexpression of α-synuclein increases Fe(II) levels in cells which then increases FoxO3a levels leading to elevated cellular sensitivity to oligomer toxicity. In contrast, β-synuclein overexpression decreases Fe(II) levels and decreases cellular sensitivity to toxic oligomers. We believe these findings provide an insight into the mechanism of dopaminergic neuronal loss in the synucleinopathies. We suggest that FoxO3a and increased Fe(II) levels are key factors in the pathway that leads to cell death.

Acknowledgments The authors thank Professor Bernard Schneider for DNA constructs containing the wild-type FoxO3a and FoxO3a-DBD constructs.

Author Contributions

D. M. Angelova, H. B. L. Jones and D. R. Brown performed the experiments. D. R. Brown designed the experiments and wrote the manuscript.

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FIGURE LEGENDS

Figure 1 Toxicity of α-syn-TO to SH-SY5Y cell lines

A SH-SY5Y cells overexpressing either α-synuclein (alpha), β-synuclein (beta) or γ-synuclein (gamma) were grown in parallel with SH-SY5Y transfected with the empty vector control (pCDNA). The cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. After this time the cells were treated with MTT and survival was assessed relative to an untreated control. Cells overexpressing γ-synuclein showed no significant difference ($p > 0.05$, Student's t test) to controls cells. However, cells overexpressing α-synuclein showed significantly more cell loss ($p < 0.05$) at concentrations between 0.75 μM and 4 μM when compared to controls. In contrast α-syn-TO was less toxic to β-synuclein overexpressing cells at concentrations of 4 μM and above. Shown are mean and s.e.m. for n=4 experiments with three replicates for each value per experiment.

B SH-SY5Y cells overexpressing either wild-type α-synuclein (alpha) or various mutants of α-synuclein were grown in parallel with SH-SY5Y transfected with the empty vector control (pCDNA). The cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. After this time the cells were treated with MTT and survival was assessed relative to an untreated control. Two mutants (H50A and 1-100) showed no significant difference to WT α-synuclein in terms of sensitivity to α-syn-TO toxicity. Both Δ2-9 and Δ2-9/H50A overexpressing cells were both significantly less sensitive to α-syn-TO toxicity

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than WT α-synuclein overexpressing cells at concentrations between 0.75 μM and 4 μM. Δ2-9/H50A overexpressing cells were not significantly different to pCDNA cells in terms of their sensitivity. This suggests this mutant abolishes the impact of increased α-synuclein expression on toxicity of α-syn-TO to cells. Shown are mean and s.e.m. for n=4 experiments with three replicates for each value per experiment.

Figure 2 Toxicity of α-syn-TO mutants

A Purified recombinant α-synuclein was generated and used to form α-syn-TO (see methods). As well as wild-type α-synuclein (WT), mutant forms of α-synuclein were also expressed and purified to generate α-syn-TO with different mutations. These mutants included an N-terminal (Δ2-9) and C-terminal mutant (Δ119-126) and a single point mutation (H50A). The cells were treated for 48 h with varying concentrations of the different recombinant α-syn-TOs in parallel. After this time the cells were treated with MTT and survival was assessed relative to an untreated control. The Δ119-126 mutation had no significant effect on α-syn-TO toxicity when compared to WT. In contrast, both H50A and Δ2-9 reduced α-syn-TO toxicity significantly at concentrations between 2 μM and 7.5 μM. The Δ2-9 mutant showed no significant difference to between different concentrations, suggesting it was not toxic at the concentrations tested. Shown are mean and s.e.m. for n=4 experiments with three replicates for each value per experiment.

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B The mutations of α-synuclein used in the experiments were tested for their ability to form β-sheets as determined by a Thioflavin T assay (ThT). Purified protein of the different mutants and wild-type α-synuclein were shaken to generate α-syn-TO. After seven days samples of the aggregated proteins were taken and reacted with ThT along with samples of the protein that had not been subjected to shaking (non-aggregated). Fluorescence at 482 nm was determined for all samples. No significant difference ($p > 0.05$) was seen for the aggregated protein of the different mutants when compared to the aggregation of the wild-type α-syn-TO. Shown are the mean and s.e.m. of four different experiments

Figure 3 FoxO3a and α-synuclein expression

Protein extracts were prepared from SH-SY5Y cells overexpressing either wild-type α-synuclein or the α-synuclein mutant Δ2-9/H50A and also from cells transfected with the empty vector (pCDNA). The protein extracts were electrophoresed on a PAGE gel and then western blotted. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), phospho-FoxO3a (p-FoxO3a), α-synuclein (α-Syn) and tubulin. α-synuclein detection was included to verify the overexpression of the protein. Densitometric analysis was then performed for the FoxO3a bands and normalized to tubulin. The average values and s.e.m. for n=4 blots were determined and plotted relative to the levels of pCDNA. A significant ($p < 0.05$) increase was observed for total FoxO3a in α-synuclein overexpressing cells but not for p-FoxO3a and no significant change was observed for either in Δ2-9/H50A cells.

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Figure 4 Toxicity of α-syn-TO and FoxO3a

SH-SY5Y cells overexpressing either wild-type FoxO3a (WT) or just the DNA binding domain (DBD) along with control cells (pCDNA) were treated for 48 h with varying concentrations of recombinant α-syn-TO. After this time the cells were treated with MTT and survival was assessed relative to an untreated control. Increased expression of wild-type FoxO3a had no effect on the survival of the cells when compared to the empty vector. In contrast DBD overexpressing cells showed significant ($p < 0.05$) difference to controls at 1.0 μM and above. The presence of DBD showed high levels of protection from α-syn-TO toxicity. Shown are mean and s.e.m. for n=4 experiments with three replicates for each value per experiments.

Figure 5 Iron and FoxO3a expression

A SH-SY5Y cells were treated with 50 μM ferrous sulphate for 24 h. Protein extracts were prepared from treated cells (Fe) and untreated controls. The protein extracts were electrophoresed on a PAGE gel and then western blotted. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), phospho-FoxO3a (p-FoxO3a), and tubulin. Densitometric analysis was then performed for the FoxO3a bands and normalized to tubulin.

B SH-SY5Y cells overexpressing α-synuclein were treated with 250 μM deferiprone for 24 h. The protein extracts were electrophoresed on a PAGE gel and then western blotted. Specific antibodies were used to detect total FoxO3a

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(T-FoxO3a), phospho-FoxO3a (p-FoxO3a), α-synuclein (α-Syn) and tubulin. Densitometric analysis was then performed for the FoxO3a bands and normalized to tubulin.

C The changes in total FoxO3a for treatments with iron and deferiprone were shown as a percentage of the control value for each experiment. Fe treatment significantly increased the levels of total FoxO3a in SH-SY5Y cells. In contrast, deferiprone significantly ($p < 0.05$) decreased the expression of FoxO3a in α-synuclein overexpressing cells. Shown are the mean and s.e.m. of four different experiments.

D The ratio of p-FoxO3a to T-FoxO3a was also determined. Densitometric values were normalized to tubulin and then the value for p-FoxO3a was divided by the value for T-FoxO3a for each group and converted to a percentage. Treatment with Fe significantly decreased the ratio of p-FoxO3a/T-FoxO3a despite the overall increase in T-FoxO3a. In contrast, deferiprone treatment led to a significant ($p < 0.05$) increase in the ratio of p-FoxO3a/T-FoxO3a. Shown are the mean and s.e.m. of four different experiments.

Figure 6 FoxO3a expression and ferrireduction

A Protein extracts were prepared from SH-SY5Y cells overexpressing either Steap-3 or β-synuclein and also from cells transfected with the empty vector (pCDNA). The protein extracts were electrophoresed on a PAGE gel and then western blotted. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), phospho-FoxO3a (p-FoxO3a), and tubulin. **B** Densitometric analysis

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was then performed for the total FoxO3a bands and normalized to tubulin. **C** The ratio of p-FoxO3a to T-FoxO3a was also determined. Shown are the mean and s.e.m. of four different experiments. * Values for Steap-3 but not β-synuclein are significantly different to the control ($p < 0.05$).

Figure 7 α-syn-TO toxicity to a β-synuclein expressing cell line

A SH-SY5Y cells overexpressing Steap-3 were grown in parallel with SH-SY5Y transfected with the empty vector control (pCDNA). The cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. After this time the cells were treated with MTT and survival was assessed relative to an untreated control. Steap-3 cells showed significantly ($p < 0.05$) greater sensitivity to the toxicity of α-syn-TO at concentrations between 2 μM and 8 μM when compared to pCDNA cells. Shown are mean and s.e.m. for n=4 experiments with three replicates for each value per experiment.

B SH-SY5Y cells overexpressing either wild-type β-synuclein (beta) or various mutants of α-synuclein were grown in parallel with SH-SY5Y transfected with the empty vector control (pCDNA). The cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. After this time the cells were treated with MTT and survival was assessed relative to an untreated control. Two mutants (H65A and 1-100) showed no significant difference to WT β-synuclein in terms of sensitivity to α-syn-TO toxicity. Both Δ2-9 and Δ2-9/H65A overexpressing cells were both significantly ($p < 0.05$) less sensitive to α-syn-TO toxicity than WT β-synuclein overexpressing cells at concentrations between 4.0

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μM and 40 μM. Δ2-9/H65A overexpressing cells were not significantly different to pCDNA cells in terms of their sensitivity. This suggests this mutation abolishes the impact of increased β-synuclein expression on protection from the toxicity of α-syn-TO to cells. Shown are mean and s.e.m. for n=4 experiments with three replicates for each value per experiments.

Figure 8 β-synuclein and Iron induced ROS

A The ratio of Fe(II) to total Fe within cells was determined for SH-SY5Y cells overexpressing either β-syn or the mutant of β-syn, Δ2-9/H65A and compared to cells transfected with the empty vector, using a commercial kit. Shown are the mean and s.e.m. for n=4 experiments. * Indicated that the values for β-syn were significantly different ($p < 0.05$) to that for pCDNA.

B The levels of ROS in the same cell lines were determined using the fluorescent compound CM-H2DCFDA. Cells loaded with CM-H2DCFDA were treated with 20 μM ferrous sulphate for 0, 1 h or 2 h. Measurements were made of cells with and without treatment with ferrous sulphate. The increase in ROS detected in ferrous sulphate treated cells above that of the untreated cells was measured and plotted as fold increase. As can be seen ferrous sulphate increase ROS levels in all cell lines when comparing the time points. The increase in ROS detected in β-syn overexpressing cells was significantly lower ($p < 0.05$) than in the other cell lines at both 1 h and 2 h time-points (*). Shown are the mean and s.e.m. for n=3 experiments.

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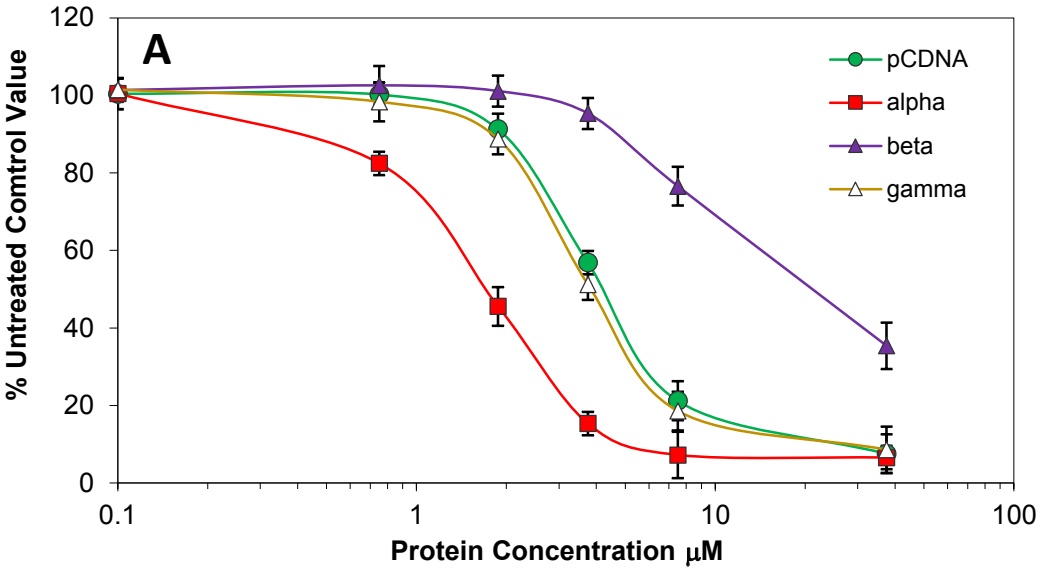

A
% Untreated Comtrol Value
120
100
80
60
40
20
0
0.1
1
10
100
Protein Concentration μM
pCDNA
alpha
beta
gamma

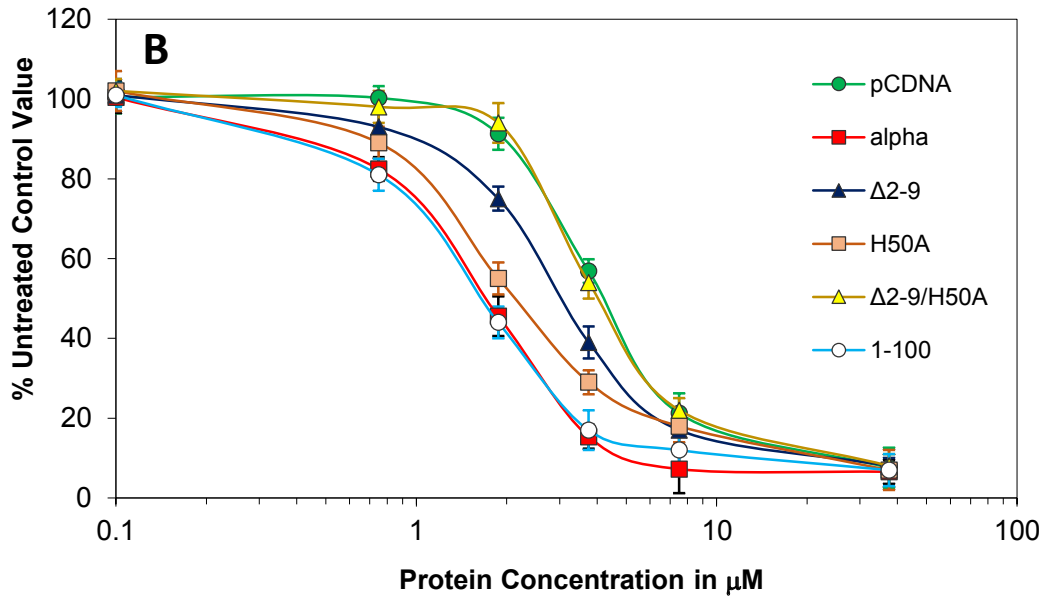

B
% Untreated Control Value
120
100
80
60
40
20
0
0.1
1
10
100
Protein Concentration in μM
pCDNA
alpha
Δ2-9
H50A
Δ2-9/H50A
1-100

Figure 1

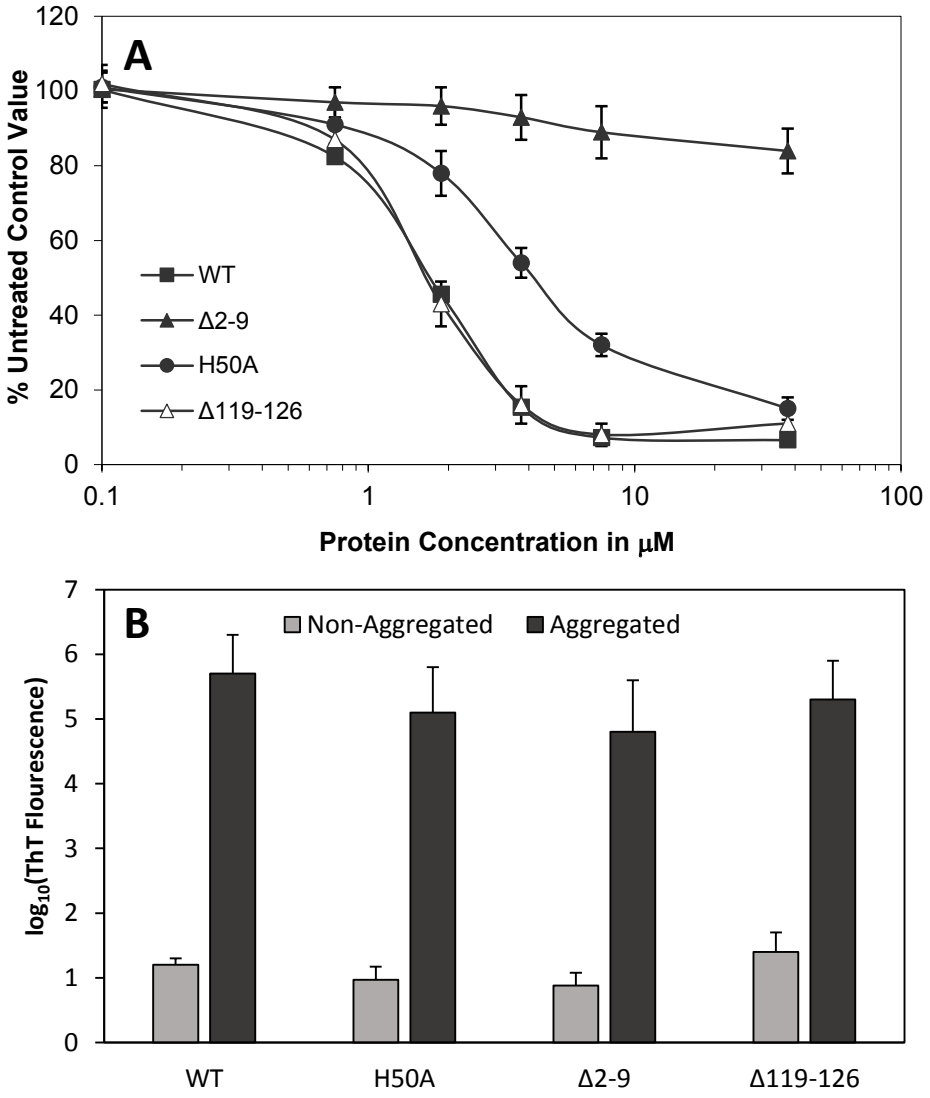
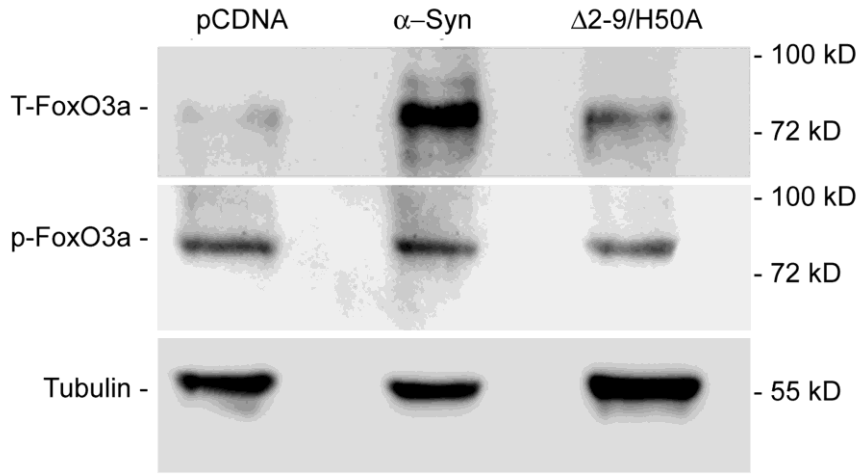

A
% Untreated Control Value
120
100
80
60
40
20
0
0.1
1
10
100
Protein Concentration in μM
WT
Δ2-9
H50A
Δ119-126
B
Non-Aggregated
Aggregated
log10(ThT Flourescence)
7
6
5
4
3
2
1
0
WT
H50A
Δ2-9
Δ119-126

Figure 2


pCDNA
α–Syn
Δ2-9/H50A
T-FoxO3a -
p-FoxO3a -
Tubulin -
- 100 kD
- 72 kD
- 100 kD
- 72 kD
- 55 kD

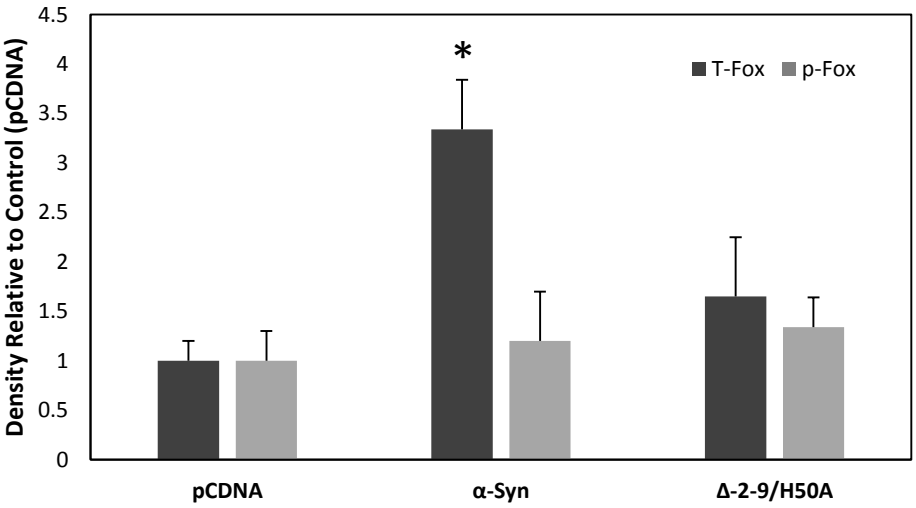

Density Relative to Control (pCDNA)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
T-Fox
p-Fox
pCDNA
α-Syn
Δ-2-9/H50A

Figure 3

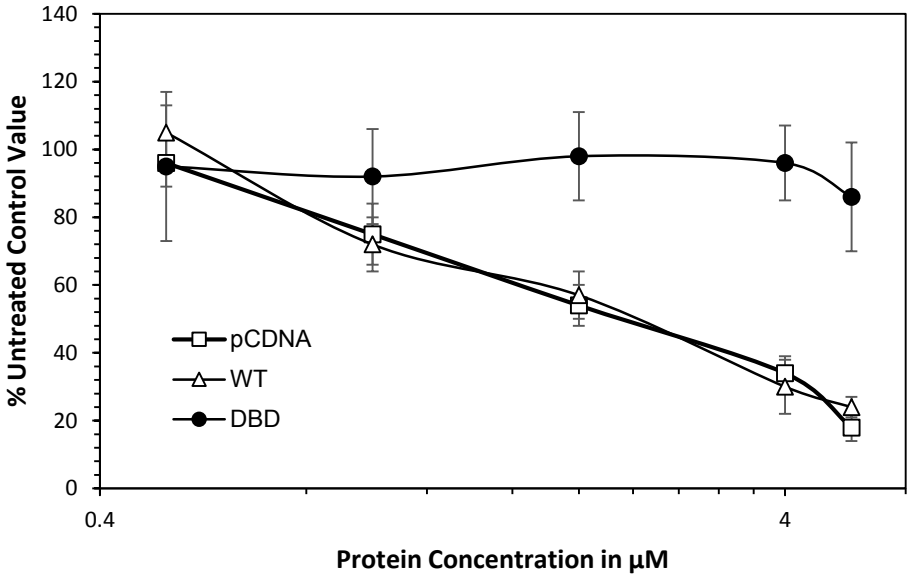

140
120
100
80
60
40
20
0
% Untreated Control Value
pCDNA
WT
DBD
0.4
4
Protein Concentration in µM

Figure 4

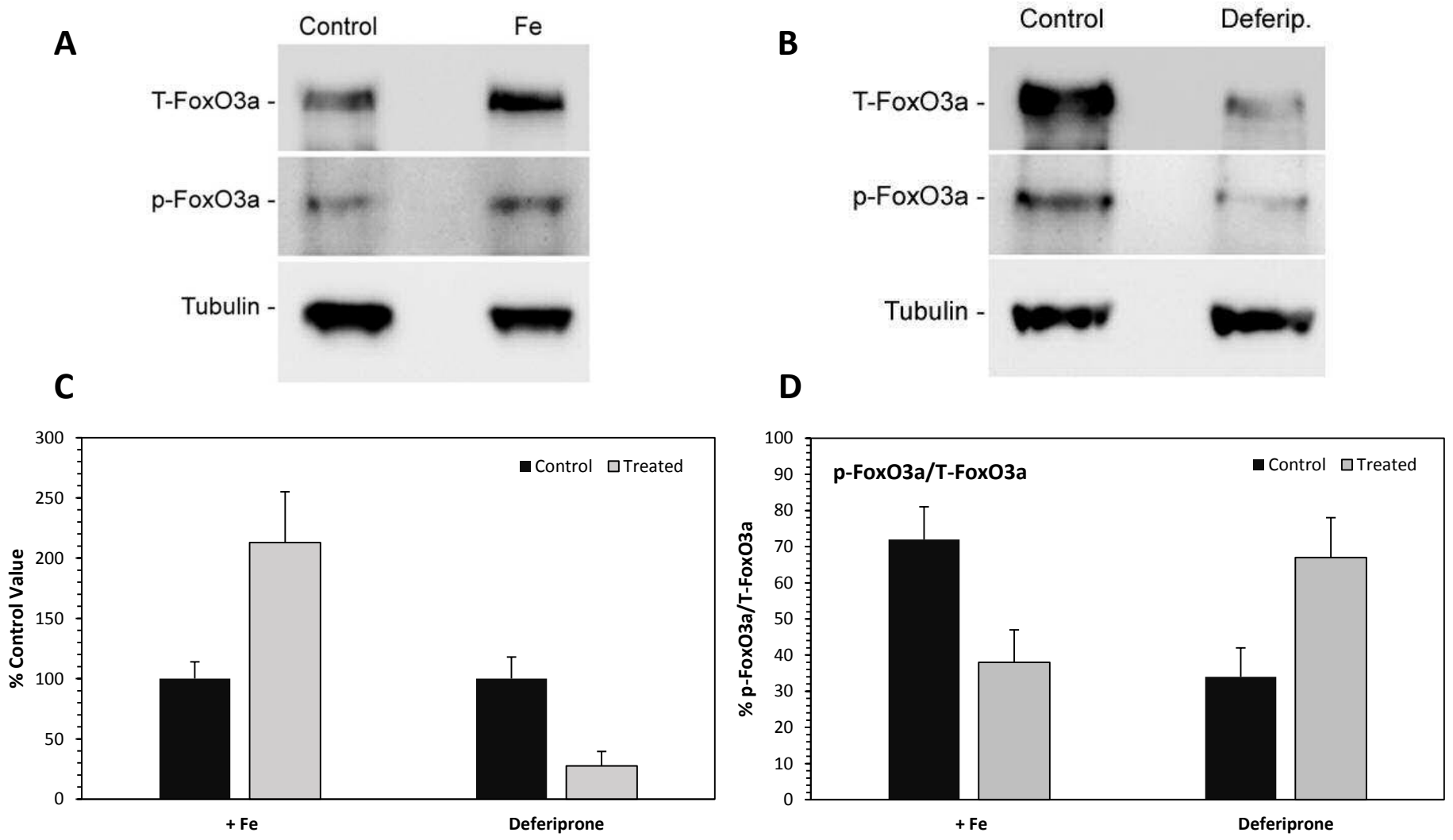

A
Control
Fe
T-FoxO3a -
p-FoxO3a -
Tubulin -
B
Control
Deferip.
T-FoxO3a -
p-FoxO3a -
Tubulin -
C
% Control Value
300
250
200
150
100
50
0
Control
Treated
+ Fe
Deferiprone
D
p-FoxO3a/T-FoxO3a
% p-FoxO3a/T-FoxO3a
100
90
80
70
60
50
40
30
20
10
0
Control
Treated
+ Fe
Deferiprone

Figure 5

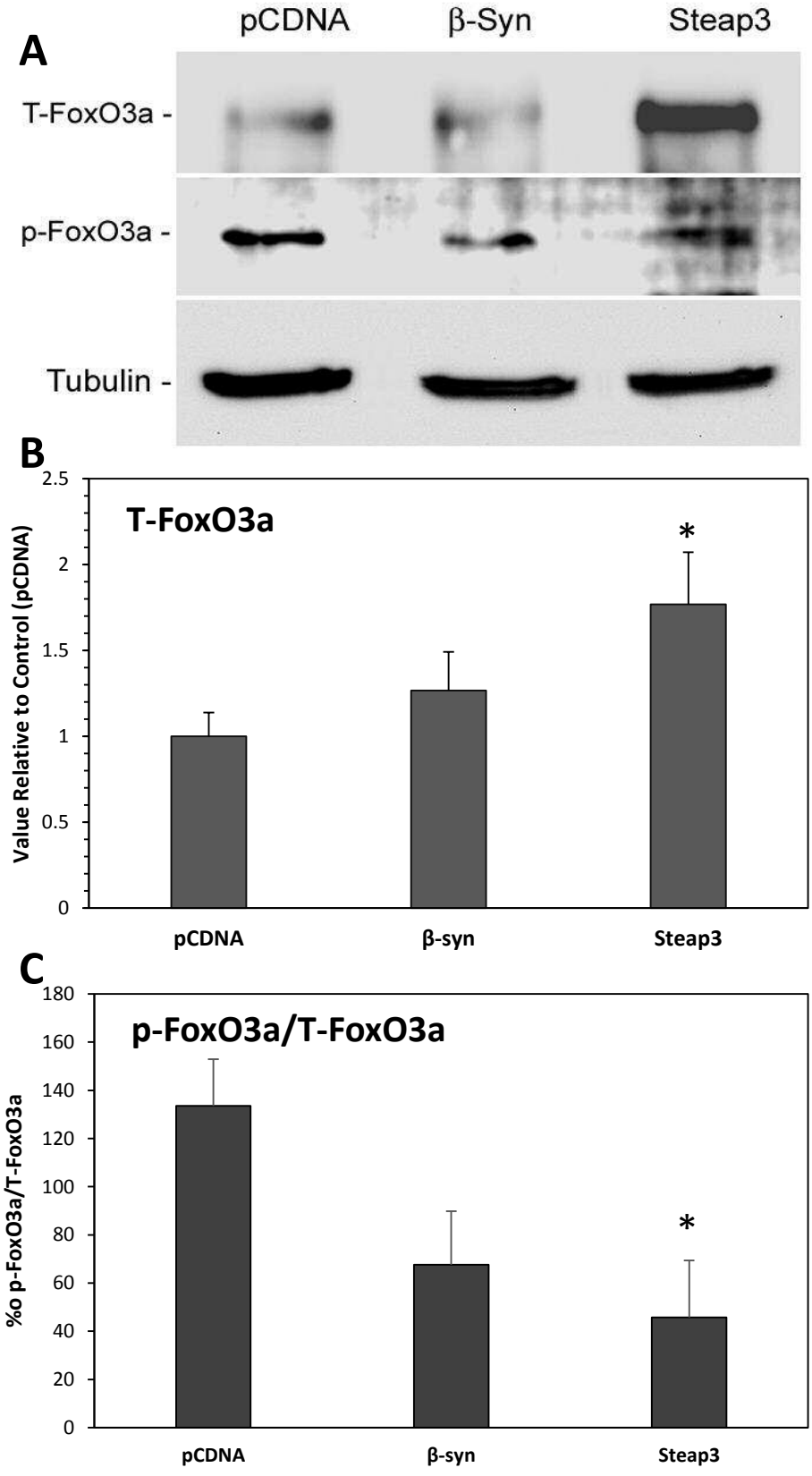

A
pCDNA
β-Syn
Steap3
T-FoxO3a -
p-FoxO3a -
Tubulin -
B
T-FoxO3a
Value Relative to Control (pCDNA)
2.5
2
1.5
1
0.5
0
*
pCDNA
β-syn
Steap3
C
p-FoxO3a/T-FoxO3a
%o p-FoxO3a/T-FoxO3a
180
160
140
120
100
80
60
40
20
0
*
pCDNA
β-syn
Steap3

Figure 6

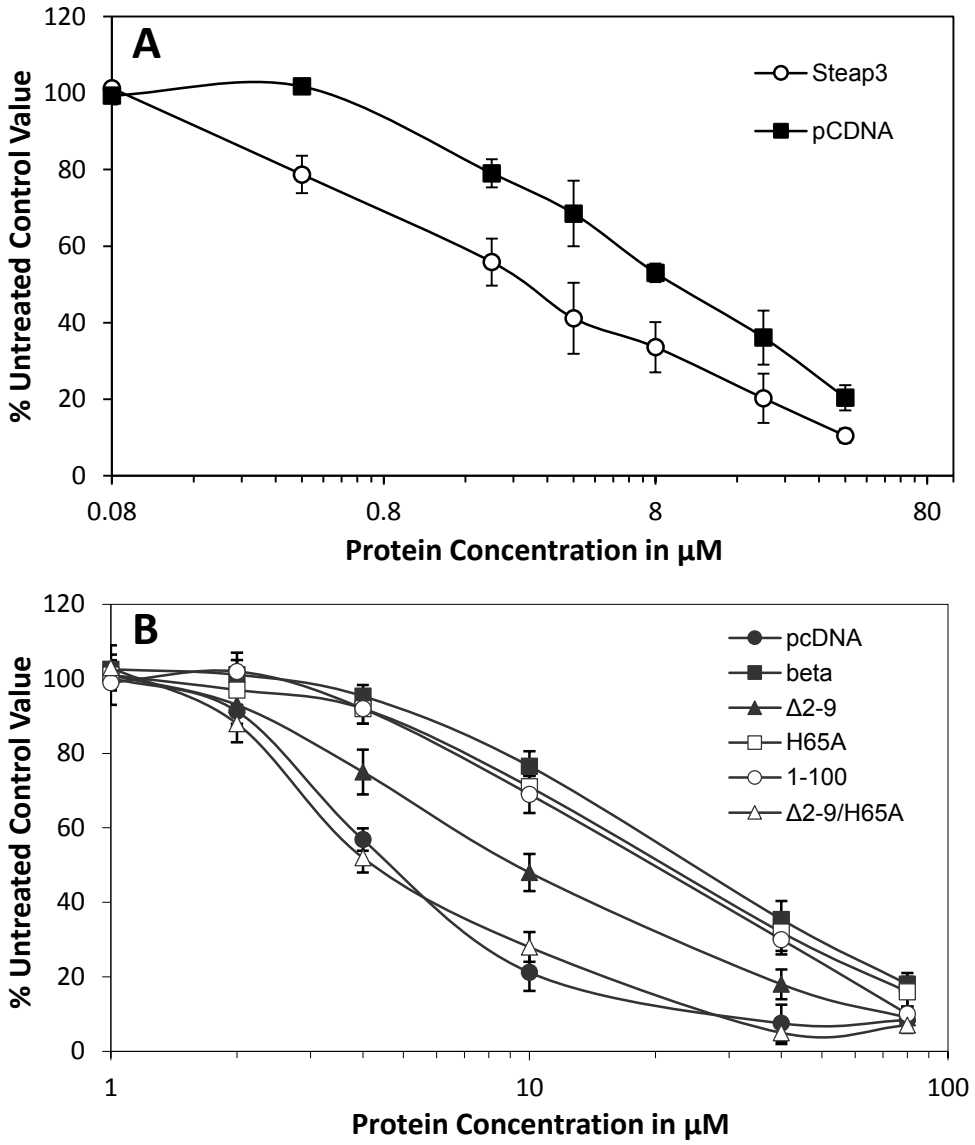

A
Steap3
pCDNA
% Untreated Control Value
120
100
80
60
40
20
0
0.08
0.8
8
80
Protein Concentration in µM
B
pcDNA
beta
Δ2-9
H65A
1-100
Δ2-9/H65A
% Untreated Control Value
120
100
80
60
40
20
0
1
10
100
Protein Concentration in µM

Figure 7

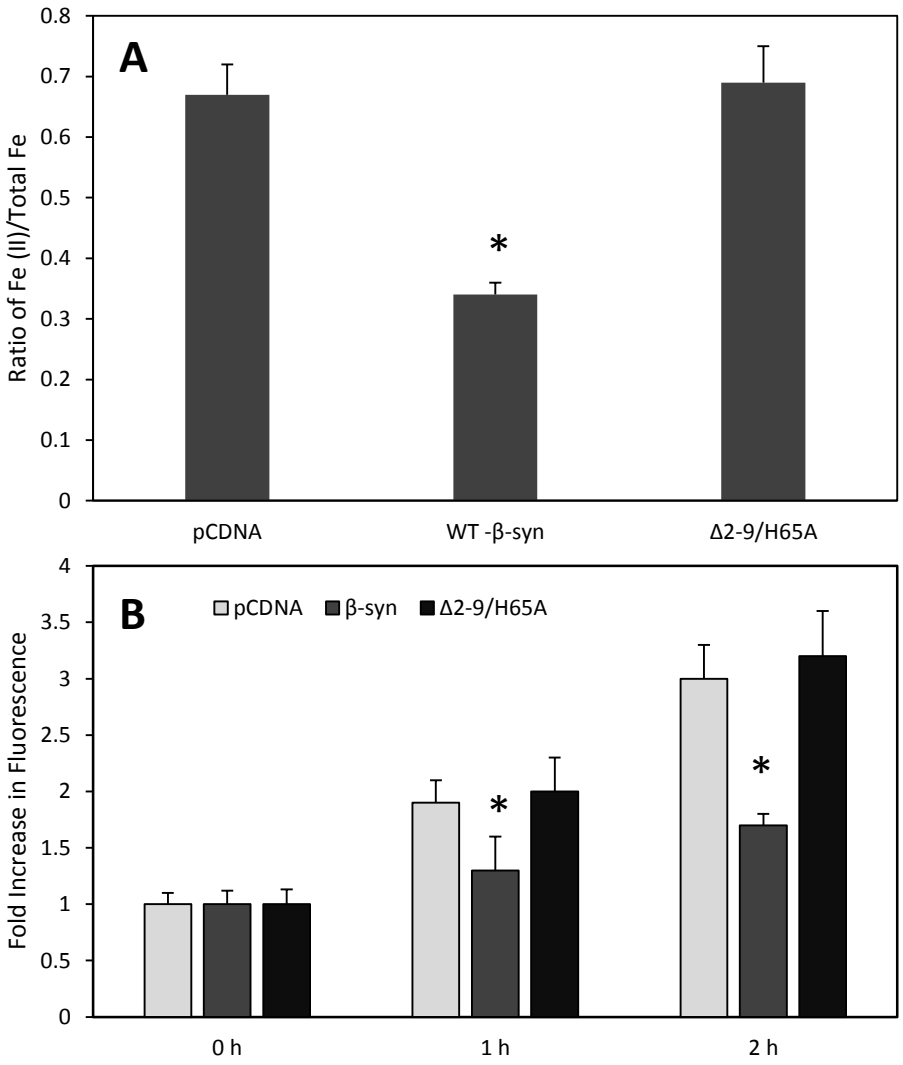

A
Ratio of Fe (II)/Total Fe
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
pCDNA
WT -β-syn
Δ2-9/H65A
B
pCDNA
β-syn
Δ2-9/H65A
Fold Increase in Fluorescence
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
0 h
1 h
2 h

Figure 8